REMARKS

Status of the Claims

Claims 24-32 are currently pending in the present application. Claims 1-23 have been canceled. Claims 24-28 are directed to the same inventions as claims 11-18. Claims 29-32, directed to a separate invention are withdrawn from consideration. Claims 24-28 are currently under examination.

Withdrawn Claims

Applicants respectfully point out that under MPEP 821.04, once a product claim is found allowable, withdrawn process claims which depend from or otherwise include the limitations of the allowable product claim will be rejoined. Claim 27 is directed to a product, and claims 29-32 are directed to methods of using the product of claim 27. Thus, once claim 27 is found allowable, claims 29-32 should be rejoined.

Moreover, MPEP 809.03 states that linking claims if allowed act to prevent restriction between inventions that can otherwise be shown to be divisible. Examples of linking claims include a claim to a product linking a process of making or using a product. Claim 27 is directed to a product, and claims 29-32 are directed to a process of using the product of claim 27. Thus, once claim 27 is found allowable, claims 29-32 which are linked to claim 27 must be rejoined and examined.

Amendments to the Claims

New claims 24-32 do not introduce prohibited new matter. The new claims provide specific embodiments of the claimed invention. Support for the new claims is summarized in the Table below.

Claim	Support
24	Original claim 11
25	Original claim 14

26	Original claim 15
27	Original claim 17
28	Original claim 18
29	Original claim 19
30	Original claim 20
31	Original claim 21
32	Original claim 22

Rejection Under 35 U.S.C. § 112, Second Paragraph

Claims 11 and 13-18 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 11 and 13-18 have been canceled and replaced with new claims 24-28. The rejection of claims 11 and 13-18 is not applicable to new claims 24-28. These new claims do not contain the language that the Office Action rejected as rendering claims 11 and 13-18 indefinite.

Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 11 and 13-18 are rejected under 35 U.S.C. § 112, first paragraph, as being enabling only for a method of producing a protein of interest comprising transforming a mutant *L. lactis* bacterium . . . ."

Claims 11 and 13-18 have been canceled and replaced with claims 24-28. Claims 24-28 are directed to a bacterial strain of the *Lactococcus* genus, wherein the *htrA* gene has been mutated so that said strain does not express a functional HtrA protease and a method of producing a protein of interest comprising introducing into the bacterial strain a nucleic acid

encoding the protein of interest and culturing the bacterial strain under conditions that would allow expression of the protein.

The Office Action asserts that the specification does not enable a method of producing a protein of interest by culturing a mutated Gram positive bacterial strain of the *Streptococcaceae* genus. Claims 24-28 do not encompass a method of producing a protein of interest comprising culturing a mutated bacterial strain of the *Streptococcaceae* genus. Moreover, the Office Action states that the specification provides enablement and support for methods of using a mutated bacterial strain of the *Lactococcus* genus which does not express a functional HtrA protease (pages 7-8). Accordingly, the invention of claims 24-28 are enabled by the specification. Applicants respectfully request withdrawal of the rejection.

Rejection of the Claims Under 35 U.S.C. § 102(b)

Claims 11, 13-16, 17, and 18 are rejected under 35 U.S.C. § 102(b) as being anticipated by Vos *et al.*

Claims 11, 13-16, 17, and 18 have been cancelled and replaced with claims 24-28. As discussed above, claims 24-28 are directed to a bacterial strain of the *Lactococcus* genus, wherein the *htrA* gene has been mutated so that said strain does not express a functional HtrA protease and a method of producing a protein of interest comprising introducing into the bacterial strain a nucleic acid encoding the protein of interest and culturing the bacterial strain under conditions that would allow expression of the protein.

The Office Action states that Vos *et al.* disclose a method of producing a protein of interest comprising culturing *Lactobacillus sp.*, which do not express a functional HtrA protease and recovering said protein exported by said strain in the culture medium. Applicants respectfully point out that Vos *et al.* do not disclose a bacterial strain that meet the limitations of the claims. The *L. lactis* bacterial strain disclosed by Vos *et al.* is *L. lactis* MG1363 which is a plasmid free strain (page 4, lines 11-14 of WO 91/02064). It is devoid of the PrtP protease since the PrtP protease is a plasmid encoded protein. However, it is not devoid of the HtrA protease, because the lactococcal chromosome encodes the HtrA protease (page 10, lines 37 of the specification). The enclosed reference of Miyoshi *et al.* confirms that the HtrA protease is expressed as a functional protease by the *L. lactis* MG1363 strain of Vos *et al.* Miyoshi *et al.* teach inactivation of the *htrA* protease gene by a single crossover recombination event using a

nonreplicative plasmid harboring an internal fragment of the MG1363 *htrA* gene (see abstract and page 3141, right column). As summarized in Table 1 (page 3142), the resulting *L. lactis htrA*-NZ9000 bacterial strain of Miyoshi *et al.* differs from *L. lactis* MG1363 of Vos *et al.* only in the disrupted *htrA* gene resulting from a single crossover recombination and the insertion of the *nisRK* genes. Accordingly, the HtrA protease is expressed by *L. lactis* MG1363 of Vos *et al.* and is a functional protease.

The Office Action states that the *L. lactis* of Vos *et al.* is transformed with expression vectors comprising mutant proteases which meet the limitations of the claims. Applicants respectfully point out that Vos *et al.* disclose expression of mutant PrtP proteases in *L. lactis*. The mutant PrtP proteases do not belong to the HtrA family. HtraA proteases are well known in the art. Pallen and Wren characterized the structure of HtrA proteases as comprising a catalytic site of serine proteases related to trypsin (see page 5, lines 15-25, of the specification). Moreover, the specification on page 8, lines 6-21, defines "HtrA protease" as "any serine protease of the trypsin type." However, the mutant PrtP proteases of Vos *et al.* are serine proteases of the subtilisin type (see page 2, lines 27-30). Thus, Vos *et al.* do not disclose a method of producing a protein of interest comprising culturing a bacterial strain of the *Lactococcus* genus which does not express a functional HtrA protease.

Accordingly, the cited reference does not teach the claimed invention, and therefore does not anticipate the claims. Applicants respectfully request withdrawal of the rejection.

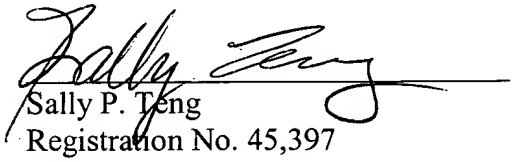
Conclusion

The foregoing amendments and remarks are being made to place the application in condition for allowance. Applicants respectfully request entry of the amendments, reconsideration, and the timely allowance of the pending claims. A favorable action is awaited. Should the Examiner find that an interview would be helpful to further prosecution of this application, they are invited to telephone the undersigned at their convenience.

Except for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which my be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310.

This paragraph is intended to be a **Constructive Petition for Extension of Time** in accordance with 37 C.F.R. 1.136(a)(3).

Respectfully submitted,
Morgan, Lewis & Bockius LLP



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Controlled Production of Stable Heterologous Proteins in *Lactococcus lactis*

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The use of *Lactococcus lactis* (the most extensively characterized lactic acid bacterium) as a delivery organism for heterologous proteins is, in some cases, limited by low production levels and poor-quality products due to surface proteolysis. In this study, we combined in one *L. lactis* strain use of the nisin-inducible promoter P_{nisA} and inactivation of the extracellular housekeeping protease HtrA. The ability of the mutant strain, designated *htrA*-NZ9000, to produce high levels of stable proteins was confirmed by using the staphylococcal nuclease (Nuc) and the following four heterologous proteins fused or not fused to Nuc that were initially unstable in wild-type *L. lactis* strains: (i) *Staphylococcus hyicus* lipase, (ii) the bovine rotavirus antigen nonstructural protein 4, (iii) human papillomavirus antigen E7, and (iv) *Brucella abortus* antigen L7/L12. In all cases, protein degradation was significantly lower in strain *htrA*-NZ9000, demonstrating the usefulness of this strain for stable heterologous protein production.

Lactococcus lactis is a gram-positive lactic acid bacterium that is widely used in the production of fermented food products, and as such, it is considered a food-grade microorganism. Experimental data and genomic analyses indicate that only a few proteins are naturally secreted in *L. lactis* (4, 32, 38), and a plasmid-free strain does not produce the extracytoplasmic protease PrtP (13). These features have drawn the attention of researchers to the potential use of *L. lactis* for secretion of proteins of biotechnological interest. Thus, *L. lactis* has been extensively engineered for production and export of heterologous proteins with high added value, such as antigens or enzymes (2, 6, 8–12, 20, 22, 23, 31, 35). For this purpose, several genetic tools have been developed for *L. lactis*, and the potential of this organism as a prokaryotic host for heterologous protein production has been confirmed (7, 9, 23, 40).

Systems that allow controlled levels of expression of foreign proteins in *L. lactis* may offer certain advantages over constitutive systems (8). The nisin-controlled expression (NICE) system (7, 18), based on a combination of the P_{nisA} promoter and the *nisRK* regulatory genes, has proven to be highly versatile (8, 17, 18) and has already been used to express different heterologous proteins (2, 6, 10, 11, 35).

Protein export to the cell surface or into the medium is a preferred means of protein expression for several biotechnological applications (9, 23). However, poor expression and proteolytic degradation of heterologous proteins are limiting factors for stable protein production in bacteria. In *Escherichia coli* and *Bacillus subtilis*, several exported proteases that are associated with turnover of both natural and foreign proteins have been described (15, 25, 26, 30, 37). In contrast to *E. coli* and *B. subtilis*, *L. lactis* has a unique extracellular housekeeping protease, HtrA (high-temperature requirement), as demonstrated by construction of an *L. lactis htrA*-IL1403 mutant strain (previously designated *htrA* [33]) and confirmed by genomic analysis (4). Studies with *htrA*-IL1403 showed that HtrA is involved in propeptide processing, maturation of native proteins, and degradation of recombinant proteins (33). These findings obtained with L. lactis have clear applications for the development of an efficient export system for high-level production of stable heterologous proteins (31a).

In this study, we constructed *L. lactis* strain *htrA*-NZ9000, an *htrA* mutant of NZ9000 (Table 1) (18). Inactivation of *htrA* was carried out by a single crossover recombination event by using a nonreplicative plasmid harboring an internal fragment of the MG1363 *htrA* gene. We examined the ability of *htrA*-NZ9000 to stabilize the following heterologous proteins that were generally degraded in wild-type (wt) *L. lactis* strains: *Staphylococcus aureus* nuclease (Nuc), a secretion reporter (21), *Staphylococcus hyicus* lipase (Lip) (10, 33, 39), bovine rotavirus nonstructural protein 4 (NSP4) (1, 11), human papillomavirus antigen E7 (2), and *Brucella abortus* antigen L7/L12 (28, 35). As we recently found that instability and/or poor yields of heterologous proteins in *L. lactis* can be overcome in part by fusion to Nuc (2, 35), the NSP4, E7, and L7/L12 proteins were fused to Nuc. Genes encoding the viral or bacterial heterologous proteins and/or the Nuc fusions were placed under control of the nisin-inducible promoter P_{nisA} and addressed for export by using the signal peptide of Usp45 (SP_{Usp45}), the predominant *L. lactis* secreted protein (38). Except for native E7, these proteins showed high levels of proteolysis in the *L. lactis* NZ9000 wt strain and were stabilized when they were produced in *htrA*-NZ9000. Our results confirm the interest in

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Characteristics	Reference
E. coli TG1	*supE hsd* Δ5 *thi* Δ(*lac-proAB*) F′(*traD36 proAB-lacZΔM15*)	14
L. lactis MG1363	Wild type, plasmid free	13
L. lactis NZ9000	MG1363 (*nisRK* genes on the chromosome)	18
L. lactis htrA-NZ9000	Em^r, *htrA* disrupted by single-crossover recombination	This study
pRV300	ColE1/Ap^r Em^r	24
pRV300:*htrA*	pRV300 derivative carrying PCR fragment (500 bp) of the *htrA* gene from *L. lactis* MG1363 (positions 47 to 547 starting at the first codon); also called pED716	This study
pSEC:Nuc	pWV01/Cm^r; expression vector containing a fusion between SP_{Usp45} and Nuc expressed under control of P_{misA}	11
pSEC:Lip	pWV01/Cm^r: expression vector containing a fusion to SP_{Usp45} in which Nuc is replaced by the *S. hyicus* Lip gene; under control of P_{misA}; previously called pJIM2093	10
pSEC:NSP4	pWV01/Cm^r; expression vector containing a fusion with SP_{Usp45} in which Nuc is replaced by the DNA fragment encoding NSP4; under control of P_{misA}	11
pSEC:LEISS:Nuc:NSP4	pWV01/Cm^r; expression vector containing a fusion with SP_{Usp45} in which LEISS:Nuc is fused to the DNA fragment encoding NSP4; under control of P_{misA}	This study
pSEC:E7	pWV01/Cm^r; expression vector containing a fusion with SP_{Usp45} in which Nuc is replaced by the DNA fragment encoding E7; under control of P_{misA}	2
pSEC:Nuc-E7	pWV01/Cm^r; expression vector containing a fusion with SP_{Usp45} in which Nuc is fused to the DNA fragment encoding E7; under control of P_{misA}	2
pSEC:Nuc-L7/L12	pWV01/Cm^r; expression vector containing a fusion with SP_{Usp45} in which Nuc is fused to the DNA fragment encoding L7/L12; under control of P_{misA}	35

combining controlled production and protein stability in one strain.

Construction of the *L. lactis htrA*-NZ9000 strain. Bacterial strains and plasmids used in this work are described in Table 1. *L. lactis* MG1363 (13) and *L. lactis* NZ9000 (18) were grown in M17 medium supplemented with 0.5% glucose (GM17), and chromosomal DNA was prepared as described previously (32). An internal 500-bp *htrA* gene fragment was PCR amplified by using the following primers designed on the basis of the genomic DNA sequence of the *L. lactis* MG1363 *htrA* gene (E. Domakova, personal communication): *htrA*5′ (5′-GGTGGAG CTATCGCACTCG-3′) for the coding strand and *htrA*3′ (5′-GGTCACCAATAGTTAACTTGCTTG-3′) for the complementary strand. This fragment was cloned into *Eco*RV-cut pRV300 (nonreplicative in *L. lactis* [24]), resulting in pED716 (referred to as pRV300:*htrA* below) (Table 1), and was established in *E. coli* TG1 (14). Recombinant clones were screened on Luria-Bertani agar plates supplemented with 150 μg of erythromycin per ml at 37°C, and plasmid DNA was isolated as described previously (3). The insert orientation was determined by dye terminator DNA sequencing analysis (ABI PRISM BigDye terminators; Applied Biosystems), and pRV300:*htrA* was then established in *L. lactis* NZ9000 by electroporation as described previously (19). Clones were isolated on GM17 agar plates supplemented with 5 μg of erythromycin per ml at 30°C. As HtrA is essential for growth at high temperatures (39°C for *L. lactis* [33]), the thermosensitivity of the *htrA* mutants was used for primary screening. For this purpose, parallel cultures were grown in liquid medium (GM17 containing erythromycin) at 30 and 39°C. *L. lactis* NZ9000 and *htrA*-IL1403 were used as positive and negative controls, respectively. After overnight growth, 30 of 35 clones did not grow at 39°C (data not shown). Inactivation of *htrA* in the 30 thermosensitive clones was confirmed by PCR amplification by using genomic DNA as the template, in which one primer hybridized to the integrated plasmid and the other primer hybridized to a region outside the *htrA* fragment used for inactivation. The oligonucleotides used were A1 (5′-GGATGGCAAAAGCTA ATATAGG-3′), A2 (5′-GGATTTGCTGTGGCTGATTTAC C-3′), AE1 (5′-GGATATTCAACAGTTTCAATTCCC-3′), and AE2 (5′-GGTTTACTTTGGCGTGTTTCATTG-3′).

Production of unprocessed Nuc in *L. lactis htrA*-NZ9000. Nuc precursor (preNuc) is synthesized as a preproprotein. During secretion, preNuc matures into NucB proprotein. NucB subsequently matures into NucA by cleavage of a 21-amino-acid propeptide in *L. lactis* (22). Poquet et al. previously showed that NucB does not mature into NucA in *L. lactis htrA*-IL1403 and concluded that HtrA is responsible for NucB-to-NucA processing (33). To confirm the *htrA*-NZ9000 phenotype, production and maturation of NucB were examined by using plasmid pSEC:Nuc (Table 1) (11). This vector contains sequences encoding the P_{nisA} promoter (7), the ribosome-binding site (RBS_{Usp45}) and the signal peptide (SP_{Usp45}) of lactococcal protein Usp45 (38), and NucB proprotein. pSEC:Nuc allowed high-level expression of Nuc due to the use of P_{nisA}, in contrast to previous findings (Nuc was produced in strain *htrA*-IL403 at a relatively low level of expression due to the use of the staphylococcal native promoter and ribosome-binding site [33]). pSEC:Nuc was introduced into *htrA*-NZ9000. Transformants were plated on brain heart infusion agar plates containing antibiotics (5 μg of erythromycin per ml, 10 μg of chloramphenicol per ml) and nisin (1 ng per ml), incubated at 30°C overnight, and then subjected to the Nuc activity assay as described previously (21). All clones displayed a Nuc$^+$ phenotype, confirming that Nuc was efficiently produced and secreted in *L. lactis htrA*-NZ9000 (21, 32). Nuc production was further analyzed by Western blotting (22, 36) by using *L. lactis* NZ9000(pSEC:Nuc) as a positive control (Fig. 1). Overnight cultures of both NZ9000 and *htrA*-NZ9000 containing pSEC:Nuc were inoculated into fresh medium at a 1:50 dilution. After 3 h of incubation (corresponding to an optical density at 600 nm of ~0.4), cultures were induced by nisin added at a final concentration of 1 ng/ml. After 1 h of induction, protein samples were prepared as previously described and analyzed

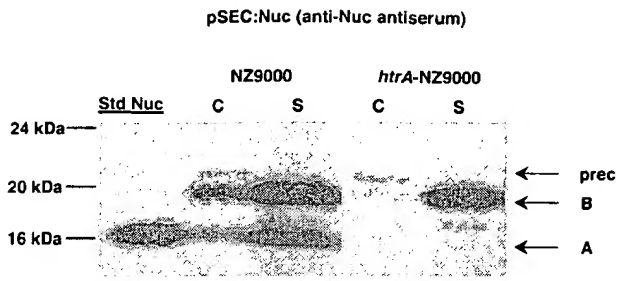

FIG. 1. Nuc production in the NZ9000 and *htrA*-NZ9000 strains. Protein extracts of induced exponential-phase cultures (1 ng of nisin per ml) of the *L. lactis* NZ9000(pSEC:Nuc) and *htrA*-NZ9000(pSEC:Nuc) strains were prepared from cell (lanes C) and supernatant (lanes S) fractions and were analyzed by Western blotting by using anti-Nuc antiserum. The migration positions of precursor forms (prec) and mature forms of both NucA (A) and NucB (B) are indicated by arrows. Commercial *S. aureus* NucA (25 ng) was used as the standard (lane Std Nuc), and molecular masses are indicated on the left.

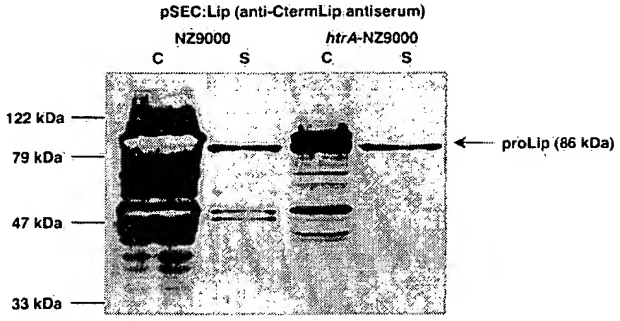

FIG. 2. *S. hyicus* lipase (Lip) production in the NZ9000 and *htrA*-NZ9000 strains. Protein extracts of induced exponential-phase cultures (1 ng of nisin ml) of the *L. lactis* NZ9000(pSEC:Lip) and *htrA*-NZ9000(pSEC:Lip) strains were prepared from cell (lanes C) and supernatant (lanes S) fractions and were analyzed by Western blotting by using antiserum directed against the Lip C terminus. The arrow indicates the position of prolipase (proLip) (86 kDa) in the cell fractions and the position of degradation products in the supernatant fractions. Molecular masses are indicated on the left.

by Western blotting by using anti-Nuc antiserum (22). The cell fraction of NZ9000(pSEC:Nuc) contained three Nuc forms (preNuc, NucB, and NucA), and the supernatant contained two major forms (NucB and NucA) and a faint band, which might have resulted from export of a C-terminal degradation product generated by cytoplasmic proteases. In contrast, the *htrA*-NZ9000 extracts contained no NucA in either cell or supernatant fractions, thus showing the lack of propeptide cleavage in this strain. The overall amounts of Nuc forms detected were higher in the wt strain than in the *htrA* mutant. Nevertheless, the yields of mature NucB were highest in *htrA* supernatant fractions. These results show that the combination of *htrA* inactivation and the NICE system allows high-level production of unprocessed NucB.

Stable production of heterologous proteins in *L. lactis htrA*-NZ9000. The heterologous proteins studied were Lip, NSP4, E7, and L7/L12. We examined production of the native and hybrid proteins (Table 1) in both strain NZ9000 and strain *htrA*-NZ9000. Protein samples were prepared from cell and supernatant fractions of induced cultures (1 ng of nisin per ml, 1 h of induction). Western blot analyses were performed by using appropriate antibodies. Production yields were compared, and quantification was performed when the appropriate standard was available.

(i) *S. hyicus* Lip. *S. hyicus* Lip has been well characterized and has potential applications in industry and medicine (10). Lip is reportedly secreted into the *L. lactis* extracellular medium in very low quantities (10) and is subject to proteolysis by HtrA during or after membrane translocation (33). Plasmid pSEC:Lip (previously called pJIM2093 [10]) was introduced into NZ9000 and *htrA*-NZ9000 and analyzed by Western blotting by using antiserum directed against the Lip C terminus (10). Considerable Lip degradation was observed in the NZ9000 cell fraction, as previously reported (10). In the supernatant fraction, one major band (86 kDa), corresponding to pro-Lip, plus several degradation products were detected (Fig. 2). In sharp contrast, Lip production was stabilized in the *htrA*-NZ9000 strain. The amounts of degradation products in the cell fraction were markedly reduced. In the supernatant fraction, a single band corresponding to pro-Lip (86 kDa) was identified (Fig. 2). The overall amounts of Lip detected were lower in the *htrA* mutant; however, the yields of intact secreted Lip in the supernatant were higher in the mutant strain.

These results confirm that degradation of Lip is due to HtrA activity (33). This hypothesis is compatible with the observation that Lip degradation occurred after translocation across the bacterial membrane (10). In conclusion, these results show that strain *htrA*-NZ9000 is capable of secreting and stabilizing full-size *S. hyicus* Lip even during high-level production.

(ii) Bovine rotavirus NSP4. Rotavirus is the major etiologic agent of severe diarrhea in infants and young children around the world (1). Its nonstructural protein, NSP4, was previously produced in *L. lactis* in fusion with SP_{Usp45} and was detected only in the cell fraction (11). In this context, two degradation products were generated in addition to the precursor NSP4 and mature NSP4 forms (11). In this study, the precursor and mature forms were also found only in the cell fraction (data not shown) in both NZ9000 strains, but in contrast to the results obtained with a wt strain (11), the quantity of degradation products was reduced in the *htrA*-NZ9000(pSEC:NSP4) strain (data not shown). Since NSP4 degradation is reduced in the *htrA* context, we suppose that the cell-associated NSP4 mature form must be exposed to the outer surface. Trying to improve NSP4 export, we designed a fusion comprising SP_{Usp45}, the synthetic propeptide secretion enhancer LEISSTCDA (referred to as LEISS below) (22), and the mature part of Nuc followed by NSP4 (Table 1). The resulting plasmid, pSEC:LEISS:Nuc-NSP4, was introduced into strains NZ9000 and *htrA*-NZ9000. LEISS:Nuc-NSP4 production was analyzed by using anti-Nuc antiserum (Fig. 3). In NZ9000(pSEC:LEISS:Nuc-NSP4), numerous degradation products were found in the cell fraction (Fig. 3). In contrast, production in *htrA*-NZ9000(pSEC:LEISS:Nuc-NSP4) resulted in significant protein stabilization in the cell fraction, and only minor degradation products appeared (Fig. 3). These results show that *htrA*-NZ9000 also stabilizes the heterologous LEISS:Nuc-NSP4 fusion. In contrast to the amounts of the two previous proteins, Nuc and Lip, the amounts of LEISS:Nuc-NSP4 are equal in the wt and *htrA* strains.

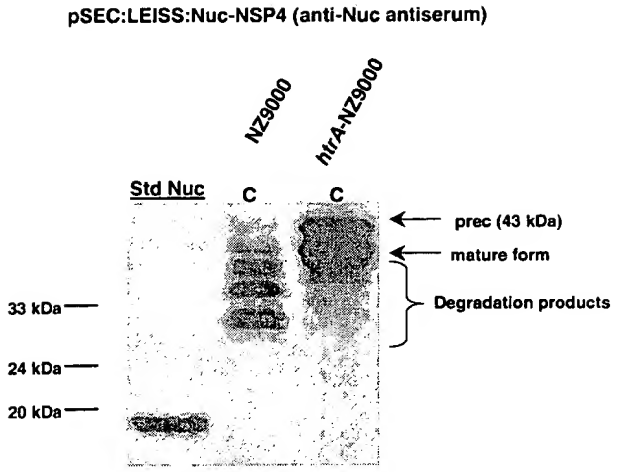

FIG. 3. LEISS:Nuc-NSP4 production in the NZ9000 and *htrA*-NZ9000 strains. LEISS:Nuc-NSP4 production was analyzed by using anti-Nuc antiserum. Protein extracts of induced exponential-phase cultures (1 ng of nisin per ml) of the *L. lactis* NZ9000(pSEC:LEISS:Nuc-NSP4) and *htrA*-NZ9000(pSEC:LEISS:Nuc-NSP4) strains were prepared from cell (lanes C) and supernatant (lanes S) fractions. The arrows and brace indicate the positions of several degradation forms, the precursor preLEISS:Nuc-NSP4 [prec (43 kDa)], LEISS:Nuc-NSP4 (mature form) (40 kDa), and degradation products. Commercial *S. aureus* NucA was used as the standard (lane Std Nuc), and molecular masses are indicated on the left.

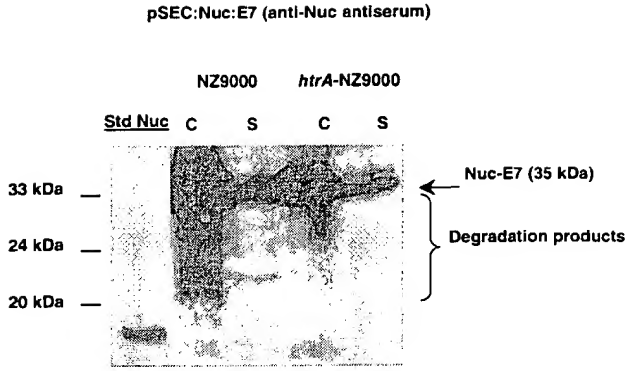

FIG. 4. Analysis of Nuc-E7 production in the NZ9000 and *htrA*-NZ9000 strains by using anti-Nuc antiserum. Protein extracts of induced cultures (1 ng of nisin per ml) of the *L. lactis* NZ9000(pSEC:Nuc-E7) and *htrA*-NZ9000(pSEC:Nuc-E7) strains were prepared from cell (lanes C) and supernatant (lanes S) fractions. The arrow and brace indicate the positions of Nuc-E7 (35 kDa) and degradation products. Commercial *S. aureus* NucA was used as the standard (lane Std Nuc), and molecular masses are indicated on the left.

Supernatant fractions were devoid of NSP4 regardless of the strain or construction tested and the antiserum (anti-Nuc or anti-NSP4) used for detection (data not shown). As previously reported (11) and confirmed here, native NSP4 is very poorly secreted. Nevertheless, our results show that the LEISS:Nuc-NSP4 precursor is processed. Furthermore, a positive Nuc activity assay (21) also suggests that the location of Nuc-NSP4 is extracytoplasmic (data not shown). The mature form of NSP4 may be associated with the cell surface because of hydrophobic domains that prevent its release into the medium. We consider it likely that both the NSP4 and Nuc-NSP4 proteins are exported but remain cell surface associated. Taken together, the results described above show that the *htrA*-NZ9000 strain is an improved host for expression of heterologous proteins, conferring high protein stability even for proteins that are poorly secreted, such as NSP4.

(iii) Human papillomavirus E7. E7 is a promising antigen candidate for development of new vaccines against cervical cancer (16, 27). Nevertheless, E7 is a labile protein (34), and this feature could be a limiting step in recombinant vaccine production (16). We recently produced E7 in *L. lactis* and found that a Nuc-E7 fusion resulted in higher production yields but lower secretion efficiency (the proportion of mature Nuc-E7 detected in the supernatant was ~10%, compared to ~95% for native Nuc [2]). However, the E7 moiety was still subject to proteolysis in a wt strain (2). Here, we tested whether secretion of Nuc-E7, as well as native E7, could be optimized in *htrA*-NZ9000.

Nuc-E7 production from pSEC:Nuc-E7 (Table 1) was examined in NZ9000 and *htrA*-NZ9000 by using anti-Nuc antiserum (Fig. 4). In the NZ9000(pSEC:Nuc-E7) cell fraction, degradation products were detected in addition to expected precursor and mature protein forms. Similarly, the supernatant contained mature Nuc-E7 plus degradation products when anti-Nuc antiserum was used. In contrast, the *htrA*-NZ9000(pSEC:Nuc-E7) strain gave rise to bands corresponding to only precursor and mature forms (Fig. 4). Thus, stable production of Nuc-E7 can be achieved in this system.

Secretion of native E7 (produced from pSEC:E7 [Table 1]) by NZ9000 and *htrA*-NZ9000 was also examined by using anti-E7 antiserum. With both strains, E7 was efficiently secreted into the medium (secretion efficiency, ~95%), and the total E7 production levels were equivalent, suggesting that native secreted E7 is not subject to HtrA degradation when it is efficiently released into the supernatant.

These results show that (i) stable Nuc-E7 is produced at a high level in the *htrA*-NZ9000 strain and is present in both cell and supernatant fractions and (ii) HtrA does not degrade native E7 but degrades Nuc-E7 hybrid protein, which is cell surface associated.

(iv) *B. abortus* immunodominant antigen L7/L12. Brucellosis, a disease caused by infection with *B. abortus*, causes abortion and infertility in cattle (5). Ribosomal protein L7/L12 is an immunodominant antigen of *B. abortus* that elicits a cell-mediated immune response and confers protective immunity in mice (28, 29). It is thus a promising candidate for development of oral live vaccines against this worldwide zoonosis. Recent results obtained in our laboratory showed that the production yields of native L7/L12 were low in *L. lactis* (35). Although better yields were obtained when L7/L12 was fused to Nuc, the Nuc-L7/L12 fusion was subject to drastic proteolysis in a wt strain (Fig. 5). We used the previously described plasmid pSEC:Nuc-L7/L12 (Table 1) (35) to analyze Nuc-L7/L12 production in NZ9000 and *htrA*-NZ9000 by Western blotting using anti-Nuc antiserum (Fig. 5). Nuc-L7/L12 precursor matured in NZ9000(pSEC:Nuc-L7/L12), indicating that a normally cytoplasmic protein can be exported. However, several degradation-size products were detected in both cell and supernatant fractions, and there were very low quantities of protein in the supernatant (Fig. 5). In *htrA*-NZ9000(pSEC:Nuc-L7/L12),

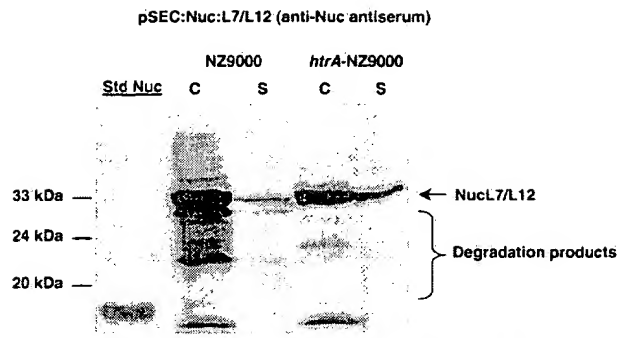

FIG. 5. Nuc-L7/L12 production in the NZ9000 and *htrA*-NZ9000 strains. Protein extracts of induced cultures (1 ng of nisin per ml) of the *L. lactis* NZ9000(pSEC:Nuc-L7/L12) and *htrA*-NZ9000(pSEC:Nuc-L7/L12) strains were prepared from cell (lanes C) and supernatant (lanes S) fractions and were analyzed by Western blotting by using anti-Nuc antiserum. The arrow and brace indicate the positions of mature Nuc-L7/L12 and a smear of degradation products. Commercial *S. aureus* NucA was used as the standard (lane Std Nuc), and molecular masses are indicated on the left.

Nuc-L7/L12 was stabilized in both cell and supernatant fractions (Fig. 5). Notably, about 10-fold more Nuc-L7/L12 was detected in the supernatant fraction of *htrA*-NZ9000 than in the supernatant fraction of NZ9000 (Fig. 5). These results demonstrate that the *htrA*-NZ9000 production strain can increase both the stability and the production yield of a hybrid protein containing a cytoplasmic moiety.

In summary, our results demonstrate that the combination of *htrA* inactivation and high-level inducible expression via the NICE system (7) leads to efficient inducible production of several heterologous proteins of medical and technological interest and can stabilize heterologous proteins that were initially degraded in a wt strain. Our results suggest that proteins that are poorly released from the cell surface are more susceptible to protein degradation than proteins that are efficiently secreted. For instance, NSP4, which remains cell associated, is subject to degradation in the wt strain. In contrast, native E7 is not degraded in the wt strain for two possible reasons: either (i) it is rapidly released and may escape HtrA-mediated degradation or (ii) it is not a substrate for HtrA. The *htrA*-NZ9000 strain may have particular applications in the stabilization of cell wall-anchored proteins; this possibility is currently being tested (Y. Dieye and J. C. Piard, personal communication). Finally, fusion of two well-secreted proteins (as exemplified here by Nuc and E7) can result in a hybrid protein that is poorly secreted (Nuc-E7 fusion) (2), suggesting that problems in protein folding result in protein degradation. The different constructions used in this study, together with our new production strain, *htrA*-NZ9000, should be valuable tools for identifying which factors other than HtrA are important for protein stability and efficient release of proteins into the medium.

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